Structural and mutational analyses of the bifunctional arginine dihydrolase and ornithine cyclodeaminase AgrE from the cyanobacterium Anabaena

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In cyanobacteria, metabolic pathways that use the nitrogen-rich amino acid arginine play a pivotal role in nitrogen storage and mobilization. The N-terminal domains of two recently identified bacterial enzymes: ArgZ from Synechocystis and AgrE from Anabaena, have been found to contain an arginine dihydrolase. This enzyme provides catabolic activity that converts arginine to ornithine, resulting in concomitant release of CO2 and ammonia. In Synechocystis, the ArgZ-mediated ornithine–ammonia cycle plays a central role in nitrogen storage and mobilization. The C-terminal domain of AgrE contains an ornithine cyclodeaminase responsible for the formation of proline from ornithine and ammonia production, indicating that AgrE is a bifunctional enzyme catalyzing two sequential reactions in arginine catabolism. Here, the crystal structures of AgrE in three different ligation states revealed that it has a tetrameric conformation, possesses a binding site for the arginine dihydrolase substrate L-arginine and product L-ornithine, and contains a binding site for the coenzyme NAD(H) required for ornithine cyclodeaminase activity. Structure–function analyses indicated that the structure and catalytic mechanism of arginine dihydrolase in AgrE are highly homologous with those of a known bacterial arginine hydrolase. We found that in addition to other active-site residues, Asn-71 is essential for AgrE’s dihydrolase activity. Further analysis suggested the presence of a passage for substrate channeling between the two distinct AgrE active sites, which are situated ~45 Å apart. These results provide structural and functional insights into the bifunctional arginine dihydrolase–ornithine cyclodeaminase enzyme AgrE required for arginine catabolism in Anabaena.

Nitrogen is an essential element in all living organisms, and how nitrogen is recycled is a key issue in nitrogen mobilization. In plants and many cyanobacteria, diverse forms of nitrogen, including atmospheric nitrogen, are converted into biologically accessible forms via assimilation (1, 2). The resulting compounds, typically glutamine and other amino acids, serve as starting materials for other metabolic pathways responsible for the synthesis of key metabolites. These organisms dynamically adjust to the environment and nutrient availability by balancing synthesis (i.e. anabolism) and degradation (i.e. catabolism) of metabolites. Bioinformatics and biochemical analyses of related genes have recently unraveled a new catabolic pathway for nitrogen–rich purine that is conserved among plants, fungi, and many bacteria, providing novel insights into nitrogen mobilization in these organisms (3, 4).

In cyanobacteria, arginine, the amino acid with highest nitrogen/carbon ratio, is a major target for nitrogen redistribution (5, 6). Arginine and aspartate are the two components of cyanophycin, a polymer for nitrogen storage in cyanobacteria (6, 7). Under nitrogen-limiting stages of cell development/growth or environmental conditions, stored arginine is liberated in an enzyme-dependent manner, making it available for nitrogen use (8, 9). However, the metabolic fate of liberated arginine has been elusive until the recent identification of ArgZ from Synechocystis (10). ArgZ contains arginine dihydrolase at its N-terminal region, which catalyzes the conversion of arginine into ornithine with a concomitant release of CO2 and two molecules of ammonia (Fig. 1A). With the identification of ArgZ, an ornithine–ammonia cycle unique to cyanobacteria was proposed (10). In this cycle, an ArgZ-dependent catabolic reaction using arginine as a substrate supplies ornithine, which then functions as a substrate in ornithine biosynthesis via four different enzymatic reactions, including a nitrogen-assimilation step. In turn, arginine produced in this cycle can be redistributed into cyanophycin for storage or processed in other catabolic pathways. Therefore, ArgZ-dependent arginine catabolism allows for its recycling, thus connecting the catabolic and biosynthetic pathways of arginine, along with a nitrogen-assimilation step. However, our understanding of the regulation of these metabolic couplings is limited. The proposed ornithine–ammonia cycle in Synechocystis has been shown to be important for nitrogen storage and mobilization because it responds dynamically to changes in nitrogen availability (10).

AgrE from diazotrophic Anabaena, a possible ortholog of ArgZ, has been shown to contain arginine dihydrolase at its N-terminal region and ornithine cyclodeaminase at its C-terminal region (Fig. 1A and Ref. 11). The latter is responsible for the formation of proline from ornithine (12), and the reaction releases one molecule of ammonia, suggesting that AgrE is a...
A bifunctional enzyme that catalyzes two sequential reactions in arginine catabolism. In another bifunctional PutA-dependent reaction (13), proline is converted into glutamate, a major nitrogen distributor. In particular, the AgrE–PutA pathway is crucial for arginine metabolism in vegetative cells. AgrE/ArgZ plays an essential role in arginine catabolism by redistributing nitrogen and carbon into coupled metabolic pathways in cyanobacteria. The enzymes AgrE and ArgZ, which have high sequence identity (Fig. 1B), exhibit relatively low sequence homology with other proteins (10, 11). This has raised interesting questions regarding the folding of each respective domain, functional aspects of each enzyme, how two independent enzymes are accommodated in one polypeptide chain, and whether communication between the two domains occurs.

In this study, we report the crystal structure of full-length AgrE in three different ligation states: an unliganded form; a ternary complex with L-ornithine, the reaction product of arginine dihydrolase, and the coenzyme NAD(H), which is required for ornithine cyclodeaminase; and a binary complex with L-arginine, the substrate for arginine dihydrolase. The structural information presented, along with kinetic analyses of mutant enzymes, provides structural and catalytic insights into agrin...
nine dihydrolase, details of an NAD(H)-binding site for ornithine cyclodeaminase, and a putative passage for substrate channeling in the bifunctional enzyme.

**Results and discussion**

**Overall structure of unliganded AgrE**

In the asymmetric unit of the crystal, full-length AgrE was arranged in a dimeric configuration, in which two monomers are related by a noncrystallographic 2-fold symmetry (Fig. 2A). Additional extensive interactions were, however, characterized between two dimers related by a crystallographic symmetry; thus, AgrE has a tetrameric conformation (Fig. 2B). Orientation of the two AgrE-NTDs in the dimer are rotated ~180° relative to each other. The tetrameric conformation of AgrE. This orientation is obtained by a ~90° rotation of A along the vertical axis followed by the placement of a second dimer. The resulting tetramer exhibits 222-symmetry along three perpendicular axes running through the center of the molecule. Consistent with the homotetramerization of AgrE, size-exclusion chromatography indicated that full-length AgrE exists as a tetrameric protein in solution (Fig. 2C). PISA analysis also indicated that AgrE potentially exists as a tetramer, with a buried surface area of ~16,900 Å² (14). Therefore, AgrE is a tetrameric enzyme with 222-fold symmetry.

The structure of monomeric AgrE at 2.06 Å resolution in an unliganded form includes three domains: the N-terminal domain (AgrE-NTD; Met-1 to Val-283), the middle domain (Glu-284 to Asp-357), and the C-terminal domain (AgrE-CTD; Leu-358 to Gly-703) (Fig. 1, A and B). Specifically, AgrE-NTD and AgrE-CTD exhibit activity for arginine dihydrolase and ornithine cyclodeaminase, respectively. Monomeric AgrE was folded into an L shape (Fig. 2D), with one end bearing AgrE-NTD and the other bearing AgrE-CTD, almost in a perpendicular orientation. The middle domain, which comprises a three-stranded β-sheet and two α-helices, was positioned at the kinked region of the L-shaped fold (Fig. 2A). Because of the 2-fold symmetric dimerization, two AgrE-NTDs are located in an opposite position relative to each other. Note that the loop in blue that connects β1 and α1 in the first β/α-propeller closes over the hollow. F, overall view of the AgrE-CTD containing ornithine cyclodeaminase. The dashed line indicates a missing loop connecting the small and large subdomains.

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2 The abbreviations used are: AgrE-NTD, N-terminal domain of AgrE; AgrE-CTD, C-terminal domain of AgrE; RMSD, root-mean-square deviation; GME, guanidine-modifying enzyme; SeMet, seleno-L-methionine; PDB, Protein Data Bank.

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**Figure 2. Structure of AgrE and its subdomains.** A, dimeric configuration of AgrE in the asymmetric unit of the crystal is shown, with each subunit in a different color. In the zoomed-in view, the protruding middle domain mediates dimerization via its three-stranded β-sheet and two α-helices. Note that the 2-fold axis of symmetry is perpendicular to the plane of the figure and runs through the dimerization interface between the two middle domains. Therefore, the two AgrE-NTDs in the dimer are rotated ~180° relative to each other. B, the tetrameric conformation of AgrE. This orientation is obtained by a ~90° rotation of A along the vertical axis followed by the placement of a second dimer. The resulting tetramer exhibits 222-symmetry along three perpendicular axes running through the center of the molecule. C, results of size-exclusion chromatographic analyses. AgrE was eluted using a Superdex 200 column (GE Healthcare) with a buffer containing 100 mM imidazole (pH 8.0). In the inset, the molecular masses of AgrE were estimated by comparing each eluted peak to molecular mass markers ranging from 43 to 669 kDa (GE Healthcare). Note that the eluted peak at 12.2 ml corresponds to a tetramer, with a void volume of 9.5 ml. D, monomeric view of an L-shaped AgrE, with each domain in a different color. The NTD (red) and CTD (blue) are oriented almost perpendicularly, with the middle domain (purple) at the kinked region. E, representative view of the AgrE-NTD for arginine dihydrolase. There are five β/α-propeller folds related by pseudosymmetry, with a central hollow. Note that the loop in blue that connects β1 and α1 in the first β/α-propeller closes over the hollow. F, overall view of the AgrE-CTD containing ornithine cyclodeaminase. The dashed line indicates a missing loop connecting the small and large subdomains.
Structure and function of AgrE

Figure 3. Binding sites of L-ornithine and L-arginine in the AgrE-NTD. A, the binding site of L-ornithine in the ternary complex is shown with a zoomed-in view. The residues within ~4.5 Å of bound ornithine are shown, with a 2Fobs − Fcalc electron-density map contoured at 1.0 σ. Possible hydrogen bonds within ~3.2 Å are indicated with dashed lines. B, zoomed-in view of the L-arginine–binding site in the structure of AgrE (C264A) complexed with L-arginine. Note that Asn-71 is closer to the NE atom of arginine than NH2.

site direction on the same plane, and two AgrE-CTDs are oriented in a face-to-face manner and also related by a rotation of 180°. The two monomers in the dimer are structurally identical, with a root mean square deviation (RMSD) of 0.91 Å for 665 Ca atoms. Tetramerization of AgrE is also achieved in a 2-fold symmetric manner such that the two AgrE-CTDs in the second dimer are inserted into the space between the two AgrE-CTDs positioned 180° apart in the first dimer. Hence, in the tetramer, AgrE-CTDs are positioned in the central region, and two AgrE-NTDs are positioned at each end (Fig. 2B).

The structural similarity search program DALI (15) revealed that a structural fold in the AgrE-NTD is highly conserved among guanidine-modifying enzymes (GMEs) of the pentein superfamily (16), with a hallmark five–β/α-propeller structure and a central hollow around the pseudosymmetrical five propellers (Fig. 2E). Three structures from the GME family are highly similar with AgrE-NTD: dimethylarginine dimethylaminohydrolase (PDB code 1h70; Z score, 30.3; RMSD, 2.1 Å) (17), succinylarginine dihydrolase (PDB code 1yni; Z score, 28.1; RMSD, 2.4 Å) (18), and arginine deiminase (PDB code 1rxx; Z score, 24.1; RMSD, 2.5 Å) (19).

Unlike the AgrE-NTD, the AgrE-CTD contains a Rossmann-fold structural feature (Fig. 2F), and the most closely related protein is lysine–oxoglutarate reductase/saccharopine dehydrogenase from Methanococcus maripaludis (PDB code 3c2q; Z score, 35.2; RMSD, 1.6 Å), which exhibits ornithine cyclodeaminase activity (11). From a structural perspective, the AgrE-CTD contains six β-stranded folds and a large α/β domain with a Rossmann fold (Val-464 and Ser-700). In both subunits, a loop of ~20 residues connecting the small and large subdomains (i.e. Lys-445A to Gly-463A and Arg-454B to Gly-463B; superscript letters represent the corresponding subunit) was missing. The large α/β domain in the AgrE-CTD contains six β–α units, and the β-strands in the β–α units form a central β-sheet with flanking α-helices (Figs. 1B and 2F). In the central β-sheet, six β-strands are positioned in a parallel orientation in the order 32, 29, 28, 34, 35, and 36, with seven flanking helices on one side and the remaining three helices on the other side of the central β-sheet. In the monomer, the surface with seven flanking helices faces the AgrE-NTD. These structural features of the monomer are homologous with those of ornithine cyclodeaminase from Pseudomonas putida; i.e. there are two subdomains, a larger subdomain with a Rossmann fold for the coenzyme-binding site, and a small subdomain in the β-stranded fold for dimerization and the substrate-binding site (12).

Active site of AgrE-NTD

The location of the active site in the AgrE-NTD was experimentally verified using the 2.4 Å resolution structure of the ternary complex of AgrE with L-ornithine and NAD+. In the ternary complex, there were no significant conformational changes relative to the unliganded form, with an RMSD of 0.36 Å for 1,351 Ca atoms. The reaction product of the AgrE-NTD, L-ornithine, was present in the central hollow of the NTD of both subunits (Fig. 3A). Specifically, L-ornithine was oriented with its side chain pointing toward the inner side of the hollow, and its α-carboxylic and α-amino groups were located near the entrance. A loop of Cys-10 to Ser-33 (Fig. 1B) connecting β1 and α1 in the first β/α-propeller appeared to close over an entrance to the hollow, although its location was essentially identical to that in the unliganded state. In addition to the residues Asn-22, Asp-65, Arg-90, Arg-139, and Tyr-167, which were within ~4 Å of the α-carboxylic and α-amino groups, the binding of ornithine was further stabilized by other interactions along the hollow. In particular, the central hollow was lined with the backbone atoms of an extended loop consisting of Ala-258 to Ala-261 after the fifth β-strand, the side chain of Lys-445C, and the backbone atoms of a seven-residue loop (i.e. Lys-445A to Gly-463A; superscript letters represent the corresponding subunit). The terminal NE atom of the α-carboxylic and α-amino groups, the binding of ornithine was further stabilized by other interactions along the hollow. In particular, the central hollow was lined with the backbone atoms of an extended loop consisting of Ala-258 to Ala-261 after the fifth β/α-propeller and the side chains of nearby residues. The terminal NE atom was also within ~4 Å of Asn-71, Tyr-167, His-168, and Cys-264. Except for Phe-68 and Tyr-167, the hollow formed by the extended loop is likely structurally important for binding the aliphatic side chain of L-ornithine. For historical reasons, we followed the structural description nomenclature for several key residues that have been defined for the GME family (16). Among these residues, Cys-264, His-168, and Glu-118 have been proposed to act as a catalytic triad: a core cysteine residue for the nucleo-
philic attack of the CZ atom in arginine, a core histidine residue to act as a catalytic acid/base for ammonia release and water molecule activation for hydrolysis, and a histidine-stabilizing glutamate residue for a dynamic hydrogen-bond network with the core histidine residue during catalysis. Another key residue is Asn-71, which functions as a lateral residue for arginine dihydrolase (16, 18).

The binding of L-arginine as a reaction substrate was observed in the 2.7 Å resolution structure of the binary complex of AgrE (C264A) with L-arginine, in which the catalytic core cysteine residue was mutated to alanine (Fig. 3B). Without any notable conformational changes even with the side-chain orientations in the hollow, the binding of L-arginine is essentially identical with that of L-ornithine in the ternary complex, except for the location of its side chain, a guanidinium group, in immediate proximity of Asp-170, which is known to function as an anterior residue that mediates interactions with the guanidinium group of substrates of the GME family (16). As a result, core Cys-264 (Ala-264 in the mutant) was located on one side of the planar guanidinium group, and the core His-168 and histidine-stabilizing Glu-118 residues were co-localized on the other side. The anterior Asp-170 and lateral Asn-71 residues were aligned in a parallel manner with the guanidinium group of L-arginine.

Activity assay for the AgrE-NTD

We first validated possible differences in activity between full-length AgrE and the AgrE-NTD (Met-1 to Val-283) (Fig. 4A). Measurements of specific activity and steady-state kinetic analysis revealed that the kinetic parameters of AgrE and the AgrE-NTD are similar, but the AgrE-NTD exhibits ~10% more activity than that of full-length AgrE, implying that the NADPH-dependent coupled assay used in this study is not significantly affected by the NAD-dependent ornithine cyclodeaminase activity of AgrE-CTD. Further analysis indicated that stoichiometric production of ammonia occurred under our assay conditions (Fig. 4B); two equivalents of ammonia were produced by the AgrE-NTD, and one equivalent was produced by arginine deiminase, a member of the arginine hydrolyase family releasing one ammonia (19). However, we were unable to characterize AgrE-CTD activity using full-length AgrE expressed and purified from Escherichia coli, as previously reported (11). Thus, all arginine dihydrolase-activity assays in this study were carried out using the AgrE-NTD or its variants.

For functional analysis of the residues in the central hollow of arginine dihydrolase, we selected 12 residues in the AgrE-NTD for site-directed mutagenesis (Fig. 4B). Without any notable conformational changes even with the side-chain orientations in the hollow, the binding of L-arginine is essentially identical with that of L-ornithine in the ternary complex, except for the location of its side chain, a guanidinium group, in immediate proximity of Asp-170, which is known to function as an anterior residue that mediates interactions with the guanidinium group of substrates of the GME family (16). As a result, core Cys-264 (Ala-264 in the mutant) was located on one side of the planar guanidinium group, and the core His-168 and histidine-stabilizing Glu-118 residues were co-localized on the other side. The anterior Asp-170 and lateral Asn-71 residues were aligned in a parallel manner with the guanidinium group of L-arginine.

Figure 4. Functional analysis of the AgrE-NTD. A, specific activity and kinetic parameters of full-length AgrE and the AgrE-NTD (Met-1 to Val-283). Each measurement in A and C was conducted in triplicate; in the case of specific activity, error bars represent standard deviations, and in the kinetic analysis, the standard error is shown in parentheses. For specific activity measurements, the reaction conditions were as follows: 2 mM arginine and 0.2 µM enzyme, and a reaction time of 30 s at 25 °C. Detailed reaction conditions are described under "Materials and methods." For kinetic analysis, the enzyme concentration was 0.2 µM. Note that specific activity was calculated based on amounts of the enzyme in nm, mainly because of differences in molecular mass between full-length AgrE and the AgrE-NTD. B, production of ammonia as a function of arginine concentration. Arginine deiminase (19), a type of arginine hydrolyase that releases one molecule of ammonia from arginine, was cloned, expressed, and purified as an N-terminal His-tagged protein. Reaction conditions were as follows: 40 µM of enzyme and a reaction time of 15 min at 25 °C. C, specific activity and kinetic parameters of the AgrE-NTD and various mutants. The reaction conditions are as described in A, but higher enzyme concentrations were used for mutants with residual activities, including 50 µM D65A, 20 µM N71A, 80 µM N71D, and 80 µM R139A.
arginine dihydrolase activity. We carried out further kinetic analysis of mutants exhibiting measurable activity in the presence of ~100- or 400-fold higher concentrations of mutant enzymes (Fig. 4C, inset). The D65A and R139A mutants showed a more than ~11–25-fold higher $k_{cat}$ and a ~488–586-fold lower $k_{cat}$, consistent with their proposed roles in mediating interactions with the α-amino and α-carboxylic groups, respectively. It is notable that N71D, a lateral Asn-71 mutant, exhibited a ~5-fold decrease in $K_m$ and a ~1,465-fold decrease in $k_{cat}$, indicating that the chemical integrity of this lateral residue is essential (see below). Taken together, these observations are consistent with the functional roles derived from structural analysis.

**The coenzyme-binding site in the AgrE-CTD**

In the ternary complex, we also characterized the binding of coenzyme NAD(H) to the AgrE-CTD by ornithine cyclodeaminase, but only one subunit in the dimer contained a binding site. Therefore, two molecules of coenzyme are present in tetrameric AgrE. The coenzyme is located at the C-terminal ends of the central β-strands and runs along the central β-sheet (Fig. 5A). Given the quality of the $2F_o - F_c$ electron-density map contoured at 1.0 σ, and possible pocket-forming residues in the vicinity of the nicotinamide moiety are shown; dashed lines indicate hydrogen bonds. Residues interacting, through the side chain, with the adenosine moiety are also indicated. Note that this figure was generated using monomeric AgrE. B, surface representation of the NAD(H)-binding site in tetrameric AgrE. Each subunit is shown in a different color, as described for Fig. 2B.

We are not sure whether these observations represent an intrinsic feature of the AgrE-CTD or are simply a crystallographic artifact. The tetrameric assembly of the AgrE-CTDs revealed some, although not extensive, intersubunit interactions at the coenzyme-binding site. We observed an extended pocket for a putative binding site for the substrate L-ornithine in the vicinity of NAD(H) (Fig. 5B). However, we were unable to observe the binding of ornithine in the vicinity of NAD(H) or ornithine cyclodeaminase activity in our experiments. It has previously been reported that AgrE purified from an *Anabaena* strain, but not from *E. coli*, exhibited ornithine cyclodeaminase activity (11). Therefore, we cannot rule out the possibility that the ornithine cyclodeaminase activity of AgrE requires another unknown factor(s). For example, SIRT6, a deacetylase with a Rossmann fold, contains an independent site for an allosteric activator (20).

**Catalytic mechanism of arginine dihydrolase in AgrE**

Members of the GME family exhibit structural and mechanistic similarity and diversity (16). Among the three functional clans in the family: hydrolase, dihydrolase, and amidotransferase, arginine dihydrolase in the AgrE-NTD is highly similar in terms of structure and catalytic mechanism with N-succinylarginine dihydrolase (18) and another hydrolase, arginine deiminase (19). Details on the catalytic mechanism have been reviewed previously (16). In these two structures, catalysis occurs via core cysteine, core histidine, and histidine-stabilizing residues, which correspond to Cys-264, His-168, and Glu-118, respectively, in AgrE (Fig. 3B). In N-succinylarginine dihydrolase, this catalytic triad has a unique position; *i.e.* the three residues are located on the opposite side of the planar guanidinium group in arginine (18). The following catalytic reaction is proposed. The core cysteine residue initiates a nucleophilic attack on the trigonal CZ atom in the guanidinium group in arginine, forming a tetrahedral adduct that is covalently attached to the enzyme. The core histidine and histidine-stabilizing glutamate residues act as a general acid–base pair to collapse the tetrahedral adduct and produce one molecule of ammonia, which is followed by the release of citrulline via hydrolysis of an intermediate.
Structural features of the catalytic triad, along with other residues in the hollow of N-succinylarginine dihydrolase (18) and arginine deiminase (19), are well-reflected in the structural analysis of arginine dihydrolase in this study (Fig. 3B). It was recently reported that citrulline could not serve as a substrate for arginine dihydrolase in ArgZ (10). Therefore, in arginine dihydrolase, there should be two successive breakages of the CZ-NH1 and CZ-NH2 bond following the formation of the tetrahedral adduct (Fig. 3B) to produce ornithine, CO₂, and two molecules of ammonia. This proposal was first suggested in a structural study of N-succinylarginine dihydrolase (18), in which a lateral asparagine (i.e., Asn-71 in AgrE) was proposed as an essential residue for dihydrolase activity (Fig. 3B). In fact, aspartate is present in arginine hydrolase and other GME family members that produce one molecule of ammonia (16). The presence of a lateral asparagine in a dihydrolase is required for a rotation of ~180° around the NE–CZ bond in the covalent intermediate. This proposed rotation could bring the second scissile CZ–NH bond, initially distant from the core histidine following the release of the first ammonia molecule, in the immediate vicinity of the core histidine for further catalysis. A lateral asparagine in a dihydrolase allows this rotation to occur because of weak interactions with the guanidinium group; this rotation does not occur with an aspartate in a hydrolase because of relatively strong interactions. Consistent with this proposal, N71A and N71D mutants in this study exhibited almost complete loss of enzyme activity, and N71D, which might have converted to a hydrolase, exhibited a higher affinity toward arginine but a significant reduction in $k_{\text{cat}}$ (Fig. 4C). An extended reaction involving the N71D mutant at a high concentration for several hours produced two equivalents of ammonia, not one. We also observed that the replacement of a lateral aspartate in arginine deiminase with asparagine yielded an inactive mutant, suggesting that there are other unknown determinants of hydrolase or dihydrolase activity.

**Substrate channeling**

AgrE is a bifunctional enzyme that catalyzes two sequential reactions. Therefore, substrate channeling between the active sites of arginine dihydrolase and ornithine cyclodeaminase has been proposed (5, 11). Substrate channeling was first proposed in a structural analysis of tryptophan synthase; namely, a tunnel connects two active sites in different subunits separated by ~25 Å (21). The structural analysis performed in this study reveals features that may allow for substrate channeling in tetrameric AgrE (Fig. 6, A and B). In the tetrameric assembly of AgrE, there is a possible empty space between the adjacent subunits. The resulting intersubunit space lined with the molecular surface of the subunits could connect the active sites of the N- and C-terminal domains. Specifically, the active-site entrance of the AgrE-NTD faces the inner side of the central region of the AgrE-CTD. In the tetramer, an intersubunit passage from the L-arginine-binding site to NAD(H) is possible, with a direct distance of ~45 Å between the Co atom of L-arginine and the nicotinamide ring of NAD(H). Except for the proposed passage from the AgrE-NTD, there are no openings around the NAD(H)-binding site in the AgrE-CTD, suggesting that the L-ornithine generated by the AgrE-NTD as a product could be channeled to the AgrE-CTD. As described previously (11), these features indicate that L-ornithine enters a catabolic pathway instead of serving as a substrate for arginine synthesis (10) under certain conditions favoring arginine catabolism, likely in vegetative cells. Finally, it is worth noting that the bifunctional enzyme PutA in the AgrE–PutA pathway also uses substrate channeling for the production of glutamate (13).

**Conclusions**

We determined three different crystal structures of the bifunctional enzyme AgrE, including an unliganded form, a binary complex with L-arginine, and a ternary complex with L-ornithine and the coenzyme NAD(H). Analysis of those structures revealed that AgrE has a tetrameric conformation, with binding sites for the substrate and product in arginine dihydrolase and a binding site for the coenzyme NAD(H) in ornithine cyclodeaminase. In particular, when AgrE was assembled as a tetramer, the AgrE-CTDs, which contain ornithine cyclodeaminase, are located in the central region, and two AgrE-NTDs, which contain arginine dihydrolase, are located at each of the two ends of the tetramer. Structural and functional analyses indicated that the AgrE-NTD is highly homologous with arginine hydrolase with respect to structure and catalytic mechanism. In addition to other active site residues, Asn-71 is...
**Structure and function of AgrE**

**Table 1**

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</tr>
<tr>
<td>α, β, γ (Å)</td>
<td>187.6, 201.7, 99.6</td>
<td>186.9, 201.1, 99.4</td>
<td>186.2, 201.5, 99.0</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
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<tr>
<td><strong>Refinement</strong></td>
<td></td>
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</tr>
<tr>
<td>R_work(%)</td>
<td>0.209</td>
<td>0.192</td>
<td>0.193</td>
</tr>
<tr>
<td>R_free (%)</td>
<td>0.247</td>
<td>0.251</td>
<td>0.245</td>
</tr>
<tr>
<td>No. of atoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macromolecules</td>
<td>10,552</td>
<td>10,605</td>
<td>10,451</td>
</tr>
<tr>
<td>Ligands</td>
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<td>62</td>
<td>24</td>
</tr>
<tr>
<td>Water</td>
<td>441</td>
<td>132</td>
<td>44</td>
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<tr>
<td>RMSD Bonds (Å)</td>
<td>0.009</td>
<td>0.009</td>
<td>0.011</td>
</tr>
<tr>
<td>Angles (°)</td>
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<td>1.18</td>
<td>1.16</td>
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<td>Ramachandran</td>
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<tr>
<td>Favored (%)</td>
<td>96.6</td>
<td>95.6</td>
<td>94.8</td>
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<tr>
<td>Outliers (%)</td>
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<td>0.2</td>
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<tr>
<td>Average B-factors (Å²)</td>
<td>31.6</td>
<td>33.2</td>
<td>23.0</td>
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<tr>
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<td>33.2</td>
<td>23.0</td>
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<tr>
<td>Ligands</td>
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<td>41.7</td>
<td>12.7</td>
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<tr>
<td>Water</td>
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<td>31.0</td>
<td>18.7</td>
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</table>

*A The numbers in parentheses refer to data in the highest resolution shell.

* The CC1⁄2 is the Pearson correlation coefficient (CC) calculated from each subset containing a random half of the measurements of unique reflection

* R_work = Σ|Fcal| − |Fccl|/Σ|Fccl|.

* R_free is the same as R_work for a selected subset (5%) of the reflections that was not included in prior refinement calculations.

essential for dihydrolase activity. AgrE-CTDs containing ornithine cyclodeaminase also contain a Rossmann-fold domain with a binding site for NAD(H), and tetrameric assembly generates a possible pocket for the substrate L-ornithine in the AgrE-CTD. Further analysis indicated that there is an empty space for substrate channeling between the active sites of the AgrE-NTD and AgrE-CTD, which were found to be ~45 Å apart. These studies provide structural and functional insights into the bifunctional enzyme AgrE.

While this manuscript was being prepared for submission, a structural and functional study of ArgZ was published (22). Many structural features of ArgZ are nearly identical with those of AgrE in this study. The study of ArgZ focuses on the structure and catalytic mechanism of arginine dihydrolase. The results have provided more direct evidence of the catalytic mechanism, in particular for a possible rotation around the NE–CZ bond.

**Materials and methods**

**Cloning and purification of AgrE**

The synthetic gene for full-length AgrE (Bioneer) was amplified using PCR, and the PCR product was cloned into a pET28 expression vector (Merck) with an N-terminal His tag. *E. coli* BL21 (DE3) cells (Novagen) harboring the resulting plasmid were cultured in LB medium at 37 °C in the presence of 50 μg/ml kanamycin. At an A_{600 nm} of ~0.6, the expression of AgrE was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside, and the cells were further cultured at 20 °C overnight. Then the cells were collected, sonicated, and centrifuged in buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM DTT, and 2 mM MgCl₂). The N-terminal His-tagged AgrE was purified via affinity chromatography using a HiTrap HP column (GE Healthcare) with buffer A and eluted with buffer B (buffer A + 500 mM imidazole). Subsequently, the His tag of AgrE was removed using tobacco etch virus protease during dialysis at 4 °C overnight with buffer A. The protein was further purified via additional affinity chromatography with buffer A, and finally via size-exclusion chromatography using a Superdex 200 column (GE Healthcare) with a buffer containing 100 mM imidazole (pH 8.0).

For the structural analysis of AgrE, a seleno-L-methionine–substituted protein was expressed in *E. coli* BL21 (DE3) in M9 minimal medium as previously described (23). The AgrE(C264A) mutant, AgrE-NTD (Met-1 to Val-283), and various other AgrE-NTD mutants were cloned and amplified via PCR using appropriate primers. The expression and purification of the proteins produced by these mutants were performed as described above for native AgrE, unless otherwise indicated.

**Crystallization and structure determination of AgrE**

Purified SeMet-AgrE, native AgrE, and AgrE(C264A) were concentrated to 6–11 mg/ml and subjected to crystallization using the sitting-drop vapor-diffusion method at 22 °C. Crystallization of the three structures described in this study was
achieved using a crystallization solution of 0.2 M sodium acetate, 0.1 M sodium citrate (pH 5.5), 10% (w/v) PEG4000, and 5% glycerol. For unliganded SeMet-AgrE, crystallization was successful with the addition of 10 mM TCEP. The binary complex of AgrE(C264A)–arginine was achieved by co-crystallizing AgrE(C264A) with 5 mM arginine. The AgrE–ornithine–NAD\(^+\) ternary complex was generated by soaking a crystal of unliganded AgrE with 5 mM ornithine and 5 mM NAD\(^+\) for 20 min.

X-ray diffraction data were collected at 100 K on Beamline 7A and 11C at the Pohang Accelerator Laboratory (Korea) with an oscillation angle of 0.5°. The collected data were processed using HKL2000 software (24), and the high-resolution cutoff was based on a CC\(_{1/2}\) statistical value (25–27). All crystals belonged to the space group C221, with two monomers in the asymmetric unit. Substructure determination and an initial phase calculation for the unliganded SeMet–AgrE structure were performed using the Phenix program (28, 29), with single-wavelength anomalous dispersion data collected at the absorption edge of selenium. Rounds of manual fitting and refinement were conducted with the programs COOT (30) and Phenix. The ternary structure of AgrE complexed with ornithine and NAD\(^+\) and the binary structure of AgrE(C264A) complexed with arginine were refined using the structure of unliganded AgrE as a starting model. During refinement, an \(F_o - F_c\) map clearly delineated the presence of arginine in the binary complex and ornithine and NAD\(^+\) at the active site of the ternary complex. Details of the data collection process and refinement statistics are presented in Table 1.

Kinetic assays of AgrE and mutants

We carried out a steady-state kinetic analysis of full-length WT AgrE, as well as of the AgrE-NTD and its various mutants. Using a coupled ammonia assay kit (Sigma), NADPH-dependent glutamate dehydrogenase converts \(\alpha\)-ketoglutarate into glutamate using ammonia liberated from an AgrE-catalyzed reaction, causing changes in the absorbance at 340 nm (18). For the enzymatic assay, AgrE variants with N-terminal His tags were purified via affinity chromatography using a HiTrap HP column followed by a desalting procedure using a HX Trap HP column. These variants were purified via affinity chromatography using a HisTrap HP column followed by a desalting procedure using a HX Trap HP column. The ternary structure of AgrE complexed with ornithine and NAD\(^+\) and the binary structure of AgrE(C264A) complexed with arginine were refined using the structure of unliganded AgrE as a starting model. During refinement, an \(F_o - F_c\) map clearly delineated the presence of arginine in the binary complex and ornithine and NAD\(^+\) at the active site of the ternary complex. Details of the data collection process and refinement statistics are presented in Table 1.

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The assays were performed at 25 °C using a UV spectrophotometer (Jasco, Japan). The assay solution contained 3 mM \(\alpha\)-ketoglutarate, 0.2 mM NADPH, 13 units/ml of glutamate dehydrogenase, and a given concentration of the AgrE variant of interest. Following incubation at 25 °C for 150 s, the reaction was initiated by the addition of arginine. The initial velocity was calculated by measuring the decrease in absorbance at 340 nm for the first 30 s and computed as the amount of ammonia measured using a standard reaction, in which the change in absorbance at 340 nm was recorded as a function of the ammonia concentration. We confirmed that all of the components in the coupled assay were present at saturating concentrations, and the measured initial velocity was dependent only on the concentration of AgrE. The NADPH molar extinction coefficient was 6,220 M\(^{-1}\) cm\(^{-1}\) at 340 nm, and SigmaPlot software was used to calculate \(V_{\text{max}}\) and \(K_{\text{m}}\).

Author contributions—H. L. and S. R. resources; H. L. data curation; H. L. software; H. L. and S. R. formal analysis; H. L. validation; H. L. and S. R. investigation; H. L. visualization; H. L. and S. R. writing—original draft; S. R. conceptualization; S. R. supervision; S. R. funding acquisition; S. R. project administration.

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References


